Purification and Properties of Ferrochelatase from the Yeast

Saccharomyces cerevisiae

EVIDENCE FOR A PRECURSOR FORM OF THE PROTEIN*

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Ferrochelatase was purified to homogeneity from yeast mitochondrial membranes and found to be a 40-kDa polypeptide with a pI at 6.3. Fatty acids were absolutely necessary to measure the activity in vitro. The Michaelis constants for protoporphyrin IX (9 × 10⁻⁸ M), ferrous iron (1.6 × 10⁻⁷ M), and zinc (9 × 10⁻⁸ M) were determined on purified enzyme preparations in the presence of diithiothreitol. However, the Kₚ for zinc was lower when measured in the absence of dithiothreitol (Kₚ ([Zn] + E) = 2.5 × 10⁻⁸ M, Kₚ ([Zn] + [E] + H₂) unchanged). The maximum velocities of the enzyme were 35,000 nmol of heme/h/mg of protein and 27,000 nmol of zinc-protoporphyrin/h/mg of protein. Antibodies against yeast ferrochelatase were raised in rabbits and used in studies on the biogenesis of the enzyme. Ferrochelatase is synthesized as a higher molecular weight precursor (Mₑ = 44,000) that is very rapidly matured in vivo to the Mₑ = 40,000 membrane-bound form. This precursor form of ferrochelatase was immunoprecipitated from in vitro translation (in a rabbit reticulocyte lysate system) of total yeast RNAs. The antibodies were used to characterize two yeast mutant strains deficient in ferrochelatase activity as being devoid of immunodetectable protein in vivo and ferrochelatase mRNA in vitro translation product. The N-terminal amino acid sequence of the purified protein has been established and was found to be frayed.

Ferrochelatase (EC 4.99.1.1) is the terminal enzyme of the heme biosynthetic pathway. It catalyzes the incorporation of ferrous iron (but not ferric iron) into the tetrapyrrole ring of protoporphyrin IX to form heme. This enzyme is associated with membrane structures in both prokaryotes (plasma membrane) and eukaryotes (inner mitochondrial membrane). It has been fairly well studied in terms of its kinetic mechanism, inhibition by N-alkylporphyrins, and metal substrate specificity and has been purified to homogeneity from various organisms (1–8), mostly by the Blue-Sepharose CL-6B affinity chromatography technique introduced by Taketani and Tokunaga (1) for the purification of rat liver ferrochelatase. All the enzymes purified from vertebrates to date have almost identical molecular masses of ~40,000 Da, but they differ in terms of their catalytic properties: the rat and chicken enzymes are activated by fatty acids, but the beef enzyme is not (1, 2, 4, 7). The only bacterial ferrochelatase which has been purified (from Rhodopseudomonas sphaeroides) is very different from the vertebrate enzymes, with a molecular mass of 105,000 Da (8).

We have initiated a series of studies to elucidate the mechanisms of regulation and function of the terminal enzymes of the heme pathway in the yeast Saccharomyces cerevisiae (6, 9, 10). Yeast is a good model organism for studies on heme synthesis since the level of intracellular heme is modulated by such physiological factors as growth in the absence or presence of oxygen or growth on fermentative or oxidative carbon sources. An efficient method has been developed to obtain heme-deficient yeast strains (11) that takes advantage of the peculiar ability of resting yeast cells incubated aerobically at pH 7.6 to synthesize large amounts of zinc-protoporphyrin (12). This physiological occurrence of zinc-protoporphyrin in yeast cells led us to investigate the role of ferrochelatase in this process. We have demonstrated that zinc and ferrous iron were competitive substrates for ferrochelatase in yeast (6) and in mammalian mitochondrial membranes (rat or human) (13, 14), with zinc incorporation being strongly inhibited by ferrous iron but not by ferric iron. These results suggested that there was some modulation of heme synthesis in vivo via the ferrous iron supply and utilization by ferrochelatase. However, metal supply may not be the only regulatory factor involved in the control of ferrochelatase activity; protoporphyrin IX delivery to the enzyme seems to be equally important in the functioning of the enzyme. This is illustrated by the observation that, although a yeast mutant which lacks protoporphyrinogen oxidase activity can accumulate and excrete protoporphyrin IX produced by nonenzymatic oxidation of protoporphyrinogen, it cannot synthesize either heme or zinc-protoporphyrin in vivo, even though ferrochelatase activity measured in vitro is normal with either iron or zinc as substrate (15, 16). This result suggested that there may be some coupling mechanisms between protoporphyrinogen oxidase activity and heme synthesis catalyzed by ferrochelatase.

Biochemical and genetic analysis of a number of heme mutants isolated in our laboratory (15) led to the characterization of strains completely lacking ferrochelatase activity. The isolation of other mutants which accumulate zinc-protoporphyrin despite synthesizing functional hemoproteins suggested that "depending on the physiological conditions, the same mutation can be expressed differently, reflecting the complexity of functioning of ferrochelatase" (17).

We have undertaken the purification and characterization of yeast ferrochelatase in order to elucidate the nature of these mutations and to study the topology and inter-relationship of ferrochelatase and protoporphyrinogen oxidase at the
level of the mitochondrial membrane. An integral part of this study was the use of antibodies raised against the purified yeast enzyme to look for the presence of a higher molecular weight precursor because despite the large number of studies devoted to ferrochelatase from various organisms, no information is yet available on the biogenesis of this enzyme. It is known that most of the proteins located in the inner mitochondrial membrane (for review, see Ref. 18) are synthesized as higher molecular weight precursors which mature during import of the protein into the mitochondria.

MATERIALS AND METHODS

Chemicals

Blue-Sepharose CL-6B, Sephadex G-25, Polybuffer exchanger 94, Polybuffers 96 and 74, and molecular mass standard proteins for Coomassie Blue staining of SDS-PAGE were from Pharmacia LKB Biotechnology Inc. Labeled molecular mass standard proteins, [35S]methionine, and rabbit reticulocyte lysate were from Amersham Corp. HEPES was from United States Biorehemical Corp. Tween 80 and all usual chemicals were from Polabo. Protoporphyrin IX disodium salt, sodium cholate, bathophenanthroline disulfonic acid disodium salt (4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt), Ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4′-disulfonic acid sodium salt), and dithizone (phenyldiazene carbothioic acid 2-phenylhydrazine) were from Serva. Anti-rabbit IgG alkaline phosphatase-conjugated antiserum and alkaline phosphatase substrates were from Promega Biotec.

Yeast Strains and Growth Conditions

The commercially available bakers' yeast *S. cerevisiae* used for purification of ferrochelatase was from Fould Springer (Maisons Alfort, France). It is a pure strain containing less than 1 × 10−9 contaminating microorganisms.

Laboratory haploid strains were used for immunorecognition of ferrochelatase, in vivo labeling, and RNA preparations. They included the wild-type strains 2D17-10B (Matα, his4, hem1-1) lacking 5-aminolevulinate synthase activity, and the ferrochelatase-deficient strains G204 (Matα, his4, hem15-1) and G221 (Matα, his4, hem15-5). All these strains were grown on complete media containing 1% yeast extract, 1% Bacto-peptone, 2% glucose (autoclaved separately), 1 g/liter Tween 80, and 20 mg/liter ergosterol. Cells were harvested during the early exponential phase of growth and used immediately.

Immunodetection of Ferrochelatase

Published procedures were used for preparing extracts from trichloroacetic acid-treated cells (19), for SDS-polyacrylamide gel electrophoresis (20), and for electrophoretic transfer of the proteins to nitrocellulose sheets (21). Incubation with the antiserum (IgG fraction) and visualization with alkaline phosphatase-conjugated antiserum (IgG fraction) were as recommended by Promega Biotec.

Total RNAs were isolated and transcribed in vitro as described (22). Immunoprecipitations were carried out on SDS-denatured proteins according to Macecchini et al. (22) with the modifications described by Urban-Grimai et al. (23).

In Vivo Labeling of Yeast Proteins

Yeast cells grown on semisynthetic medium deprived of sulfate ions (19) were harvested during the early exponential growth phase and resuspended in 25 mM sodium citrate buffer, pH 6.5, containing 10% glucose, [35S]methionine (500 μCi/ml), and 10 μg/ml tyrosine to increase methionine uptake efficiency (24). After different incubation times (15 s to 1 h), aliquots were taken and diluted with cold methionine. The cells were broken with glass beads, proteins were precipitated with 20% trichloroacetic acid, and samples were analyzed for immunoprecipitable ferrochelatase. Since ferrochelatase represents only 0.005% of the total yeast proteins, two consecutive immunoprecipitations were required for analysis of the labeled material. The first immunoprecipitated pellet was solubilized in the SDS-PAGE sample buffer, and an aliquot was analyzed by electrophoresis. The remaining labeled proteins were precipitated with trichloroacetic acid (20% final concentration), resolubilized with 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100, processed again for immunoprecipitation, and analyzed by SDS-PAGE.

Protein Assays

Protein concentrations were determined (i) as described by Lowry et al. (25) using bovine serum albumin as a standard during the initial steps of purification and (ii) by the method of Bradford (26) during the final steps of purification. Since detergents, especially Tween 80, interfere with these assays, proteins were first precipitated with acetic acid/acetone (1:5, v/v) and dissolved in 0.1 N NaOH.

Ferrochelatase Assay

Ferrochelatase was assayed spectrofluorometrically by measuring the rate of protoporphyrin IX disappearance (iron-chelatase activity) or zinc-protoporphyrin formation (zinc-chelatase activity) (6,13). Protoporphyrin and zinc-protoporphyrin were quantified with a JenK-Yvon J13-D spectrophotometer equipped with a thermostated cell holder, a red-sensitive Hamamatsu R924 photomultiplier tube, and a slit system set as follows: 2 nm each for entry and exit for the excitation monochromator and 2 nm for entry and 20 nm for exit for the emission monochromator. The maximum excitation and emission wavelengths for protoporphyrin IX and zinc-protoporphyrin were 410-432 and 420-587 nm, respectively, in the buffer systems used.

For routine ferrochelatase assays (monitoring chromatographic processes), the incubation mixture consisted of 0.1 M Tris-HCl, pH 7.6, containing 1 μM protoporphyrin, 5 μM Zn++, 1 mM palmitic acid (see "Results"), and 0.3 mg/ml (final concentration) Tween 80 to ensure maximum fluorescence signal, keeping protoporphyrin in a monomeric state all during the incubation. Protoporphyrin stock solution was prepared as 50 μM protoporphyrin disodium salt dissolved in 0.1 M Tris-HCl, pH 7.6, containing 1% (w/v) Tween 80. Zinc was prepared as a 0.3 mM stock solution of ZnSO4·H2O dissolved in distilled water. Palmitic acid was prepared as a 20 mg/ml stock suspension sonicated in water (3 × 30 s, 50 kHz). Ferrochelatase activity was initiated by addition of the enzymatic fraction and followed by time-dependent measurement of fluorescence in 1-ml glass tubes. Under these conditions of wavelength pairs and initial velocity measurements, fluorescence transfer was observed between protoporphyrin and zinc-protoporphyrin.

For all kinetic studies on yeast ferrochelatase, the incubation medium (3 ml in 4-ml fluorometric cuvettes with magnetic stirring) was designed to minimize the amount of endogenous metals, mainly zinc, which contaminated most of the commercially available chemicals. It was made of 50 mM HEPES, pH 7.50, containing 0.5 mg/ml Tween 80, and 1 mM palmitic acid (plus 5 mM dithizone, which contaminated most of the commercially available chemicals). The remaining labeled proteins were precipitated with trichloroacetic acid; protoporphyrin IX, iron, and zinc concentrations were varied as described in the figure legends. HEPES buffer was freed of zinc as described by Nicholas (27), except that dithizone was used instead of 8-hydroxyquinoline. Since Tween 80 and palmitic acid are soluble in chloroform, another method was designed to remove zinc by using the hydrophobic chelator Ferrozine. Tween 80 and palmitic acid were solubilized in chloroform, and the organic phase was washed three times with a 1 mM aqueous solution of Ferrozine. The removal of the aqueous phases was followed by evaporation of the chloroform under a stream of nitrogen. Tween 80 (1%), w/v) was solubilized in 50 mM zinc-free HEPES buffer, pH 7.50, protoporphyrin IX stock solution, was prepared as 50 μM protoporphyrin disodium salt dissolved in the above Tween 80-containing buffer. Palmitic acid (20 mg/ml) was solubilized in dimethyl sulfoxide, which allows stable emulsions of the fatty acid in the aqueous assay medium. Ferrous iron (FeSO4·H2O 0.1 mM final concentration) was prepared in anhydrous dimethyl sulfoxide containing diethiothreitol (0.3 M). Dimethyl sulfoxide (1-5% v/v) did not interfere with the measurement of ferrochelatase activities. Aqueous diethiothreitol stock solution was prepared at 0.3 M in 50 mM zinc-free HEPES buffer, pH 7.50. In all the experiments, Tween 80, dimethyl sulfoxide, and diethiothreitol concentrations were kept constant to avoid additional variables in the assays.
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Preparation of Ferrochelatase from Yeast Cells

All operations were carried out at 4°C. The purification procedures described below gave identical results when used with either commercially available yeast cells (kilograms of starting material) or lab-sensitized strains (grams of starting material).

**Step 1: Preparation of Cell-free Extracts—**Yeast cells (5 kg of commercial-grade packed cells, wet weight) were washed twice in distilled water and once in 0.1 M potassium phosphate buffer, pH 7.6, containing 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride (Buffer A). The cells collected by centrifugation for 15 min at 4,000 × g were resuspended in Buffer A at a concentration of 50-60 mg of proteins/ml, homogenized in a Potter-Elvejhem homogenizer, and frozen as 50-ml aliquots at -80°C. They could be kept for months without loss of ferrochelatase and protoporphyrinogen oxidase activities.

**Step 2: Preparation of Membrane Fraction Enriched in Mitochondrial Membranes—**The cell homogenate was centrifuged at 50,000 × g in a continuous flow, air-driven refrigerated centrifuge (Sharples-Stokes Div. Model T41-24). The flow rate was 2 liters/h. The pellet was resuspended in Buffer A at a concentration of 50-60 mg of proteins/ml, homogenized in a Potter-Elvejhem homogenizer, and frozen as 50-ml aliquots at -80°C. They could be kept for months without loss of ferrochelatase and protoporphyrinogen oxidase activities.

**Step 3: Solubilization of Membranes—**In a typical experiment, eight aliquots of frozen membranes (400 ml) were thawed and washed by centrifugation for 60 min at 150,000 × g. The pellet was resuspended in the initial volume in Buffer A and homogenized. Concentrated Tween 80 (10%, v/v, in Buffer A) was then added to the membrane suspension to give a final detergent/protein ratio of 0.5 (v/w) and a final volume of 500 ml. The mixture was sonicated (8 × 1 min) at 100 kHz and kept at 4°C for 3 h with magnetic stirring. Insoluble material was then removed by centrifugation for 90 min at 150,000 × g. The supernatant containing ferrochelatase and protoporphyrinogen oxidase activity was saved.

**Step 4: Blue-Sepharose Chromatography—**Glycerol (20% final concentration) was added to this supernatant. The resulting solution was loaded onto a 2.6 × 40-cm column packed with Blue-Sepharose CL-6B equilibrated in 25 mM Tris-HCl, pH 8.0, containing 20% (v/v) glycerol and 1% (w/v) Tween 80 (T/G/Tween buffer). Ferrochelatase was eluted from the column after a series of stepwise buffer changes in which both the ionic strength and the nature of the buffer varied. The resulting fractions were assayed for ferrochelatase activity.

**Step 5: Chromatofocusing—**The concentrated solution of ferrochelatase was loaded onto a 1 × 15-cm column packed with Polybuffer exchanger 94 equilibrated in 25 mM imidazole HCl, pH 7.4, containing 20% (v/v) glycerol and 0.1% (w/v) Tween 80. Ferrochelatase was eluted with a linear gradient of pH produced by a solution of 12.5% Polybuffer 74, 20% glycerol, and 0.1% Tween 80 adjusted to pH 6.0 with 6 N HCl. The flow rate was 25 ml/h, and 2-ml fractions were collected. Active fractions were pooled and concentrated as described above.

The resulting enzyme preparation was apparently homogeneous when analyzed by SDS-PAGE. The purified enzyme was stored at −80°C as a 0.1-mg protein sample. There was no loss of activity for the longest period of time as a 1 ml sample stored at −80°C for 1 year as long as palmitate was present in the assay medium (see "Results").

**Units of Activity—**One unit of ferrochelatase activity is the amount of enzyme needed to catalyze the formation of 1 nmol of metalloprotoporphyrin/h at 30°C in the standard assay system.
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is also somewhat more complex than that described by Taketani and Tokunaga (1). It was absolutely necessary to remove first the Tween 80 and then the KCl before running the cholate and salt buffers onto the column, otherwise the enzyme was eluted along with several other contaminating proteins. The enzyme eluted with T/G buffer, and 1% cholate plus 1 M KCl appeared to be fairly pure. A single contaminant polypeptide of $M_r = 67,000$ was detected on SDS-PAGE. Final purification of ferrochelatase was achieved after a chromatofocusing step in which the enzyme was eluted at pH 6.3. It was found to be absolutely necessary to run this column with a minimum amount of detergent (0.1% Tween 80). The absence of detergent resulted in total loss of the gel binding capacity no matter what the pH was of the equilibration buffer used. Glycerol did not interfere with the pH gradient forming compounds of the elution buffer. The enzyme, purified to homogeneity, displayed a single band on SDS-PAGE with an apparent molecular mass of 40,000 Da (Fig. 1). The same purification procedure was successfully used to purify the enzyme from laboratory-grown wild-type (D273-10B) and heme-deficient (G204) yeast strains. The purified enzyme was stable for months when stored in 20% glycerol/Tris buffer at -80 °C. Ferrochelatase represented less than 0.005% of the total proteins in a wild-type strain (Table 1). The absorption spectrum of the purified enzyme showed only the classical absorption maximum at 280 nm; its optimum pH was 7.6, as reported for the membrane-bound enzyme (6).

**FIG. 1.** Electrophoretic profile (Coomassie Blue staining) of purified yeast ferrochelatase on SDS-10% PAGE. Lane A, membrane proteins from wild-type yeast strain FL200; lane B, membrane proteins from ferrochelatase-deficient strain G214 (150 µg of total proteins each); lane C, molecular mass standard proteins (in kilodaltons) (3 µg/band); lane D, purified yeast ferrochelatase (10 µg).

**FIG. 2.** Effect of palmitic acid concentration on zinc-chelatase activity of purified ferrochelatase (10 µg/assay). Protoporphyrin IX and zinc concentrations were 1 and 5 µM, respectively.

**FIG. 3.** A, inhibitory effect of dithizone on zinc-chelatase activity of ferrochelatase. Ferrochelatase (3 µg) was assayed in the routine
Catalytic Properties of Purified Enzyme—As previously described for the rat liver enzyme (1), purified yeast ferrochelatase has an absolute requirement for fatty acids to be active in vitro. The dose-response curve for ferrochelatase activity as a function of palmitic acid concentration was consistently found to be sigmoid (Fig. 2).

The Michaelis constants were measured on the pure enzyme for ferrous iron, zinc, and protoporphyrin IX. Two main problems arose that rendered difficult the kinetic studies of zinc-chelatase and iron-chelatase activities of ferrochelatase. The first one lay on the presence of enough zinc in most of the purest chemicals available (i) to saturate the enzyme during zinc-chelatase measurements and (ii) to inhibit competitively iron incorporation. This is illustrated in Fig. 3A where zinc-chelatase activity was assayed in the routine assay medium (see "Materials and Methods") containing increasing concentrations of the zinc-chelating agent dithizone. The use for zinc-chelatase activity was assayed in the routine assay medium with and without dithiothreitol (DTT), DTT being also present in the assay medium, zinc-chelatase activity was undetectable (with 80 nM endogenous zinc). Therefore, this allowed the measurements of the $K_m$ values for ferrous iron ($1.6 \times 10^{-7}$ M) and protoporphyrin IX ($9 \times 10^{-8}$ M) in the iron-chelatase assay for ferrochelatase (Fig. 3, B and C). When the Michaelis constant for zinc was determined in the presence of 5 mM DTT, the $K_m$ value was very different from that measured in the absence of DTT ($9 \times 10^{-6}$ versus $2.5 \times 10^{-7}$ M; Fig. 3D), with the $K_m$ for protoporphyrin being unchanged (data not shown). The fact that DTT interferes with zinc incorporation might result in either a complexation of zinc by DTT or an effect of DTT on the enzyme itself. The first hypothesis seemed unlikely since the accessibility of zinc to the chelator dithizone was the same with and without DTT.

To test the second hypothesis, we used the sulphydryl group inhibitors $p$-chloromercuribenzoate and iodoacetamide during zinc-chelatase assay measurements. As shown in Fig. 3E, both reagents were dose-dependent inhibitors of zinc-chelatase activity of ferrochelatase, $p$-chloromercuribenzoate being 1000 times more reactive than iodoacetamide. These results suggested the involvement of some sulphydryl groups in ferrochelatase activity. In addition, the iron-chelatase activity of ferrochelatase previously inhibited by $p$-chloromercuribenzoate was restored by 5 mM DTT, as already shown for the rat liver enzyme (1).

N-terminal Amino Acid Sequence Analysis—The purity of the protein was ascertained by establishing its N-terminal amino acid sequence. A 20-residue N-terminal sequence was determined for the delipidated enzyme by automated Edman degradation (Table II). It was found, in two independent preparations and sequence determinations, that the N-terminal sequence is frayed, with 75% of the chains starting with Asn-Ala-Glu-Lys-Arg and the remaining 25% of chains starting with the second amino acid, Ala-Glu-Lys-Arg...

**Immunochemical Characterization of Ferrochelatase**—The molecular mass of ferrochelatase (40 kDa) was found to be identical for the purified enzyme and for the enzyme detected by the immune replica method in total protein extracts prepared from various wild-type or heme-deficient laboratory strains (Fig. 4). The two exceptions were strains G214 and G221 (allelic to G214), described as deficient in ferrochelatase activity in vitro. These strains were also found to be completely devoid of immunoreactive protein. Thus, the relative molecular mass of native ferrochelatase is 40 kDa.

When total RNAs (from all the yeast strains tested) were translated in a rabbit reticulocyte lysate system, the immunoprecipitated newly synthesized ferrochelatase exhibited a higher molecular weight ($M_s = 44,000$). This higher molecular weight polypeptide was also detected in pulse-labeled cells, showing that it is not an immunoprecipitation artifact, but is a precursor form of ferrochelatase (Fig. 5). When total RNA from strains G214 and G231 were tested for in vitro translation, the pattern of total proteins synthesized was identical to that obtained with other strains, but no ferrochelatase was detectable (data not shown).

**Table II**

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<th>N-terminal amino acid sequence analysis of delipidated yeast ferrochelatase</th>
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| A | 75% Asn-Ala-Glu-Lys-Arg-Ser-Pro-Thr-Gly-Ile-
| B | 25% Asn-Ala-Glu-Lys-Arg-Ser-Pro-Thr-Gly-Ile-Val-
| II |
| A | Val-Leu-Met-Asn-Met-Gly-Gly-Pro-Ser-Lys-
| B | Leu-Met-Asn-Met-Gly-Gly-Pro-Ser-Lys-
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FIG. 4. Immunodecoration of ferrochelatase after electro-transfer of trichloroacetic acid-precipitable total proteins from various yeast strains after resolution on SDS-10% PAGE. Lane A, purified ferrochelatase (0.1 µg); lane B, wild-type strain D273-10B; lane C, ferrochelatase-deficient strain G231, lane D, ferrochelatase-deficient strain G214; lane E, 5-aminolevulinic synthase-deficient strain G204; lane F, protoporphyrinogen oxidase-deficient strain G122. Anti-ferrochelatase antibodies were used at a 3000-fold dilution and revealed by using alkaline phosphatase-conjugated anti-rabbit IgG secondary antibodies.

FIG. 5. Autoradiography of labeled ferrochelatase immunoprecipitated from in vitro translation products of 20 µg of total yeast RNAs, strain D273-10B (lane B), and in vivo labeling (lane C) after two consecutive immunoprecipitations. In lane A are labeled molecular mass markers. P, ferrochelatase precursor; M, mature form of the enzyme.

immunoprecipitable (data not shown), suggesting the absence (or a great instability) of the ferrochelatase mRNA.

When ferrochelatase was immunoprecipitated from cells metabolically pulse-labeled, the precursor form of ferrochelatase was detectable only for very short labeling times (15 and 30 s); longer labeling times led to immunoprecipitation of the mature form of the enzyme (Fig. 6A; see details in Fig. 6B). As shown in Fig. 6, many contaminating polypeptides were coprecipitated with ferrochelatase, although the same immune serum detected only ferrochelatase by Western blotting or when used for precipitation of in vitro synthesized ferrochelatase (Figs. 4 and 5). This might suggest that in vivo labeled ferrochelatase is first precipitated as part of an aggregate representing the surroundings of the enzyme in situ. When these immunoprecipitated proteins were resolubilized in detergent-containing buffer and precipitated a second time, a single polypeptide chain corresponding to ferrochelatase was detectable (Fig. 6C).

FIG. 6. Autoradiography of labeled ferrochelatase analyzed by SDS-10% PAGE after various times of in vivo labeling and immunoprecipitation of equal amounts of processed cells. A, first immunoprecipitation (7-day exposure); B, partial view of the autoradiogram exhibiting ferrochelatase (F) precursor (P) and the mature form of the enzyme (M) (15-day exposure); C, second immunoprecipitation (SDS-10% PAGE; 7-day exposure).

DISCUSSION

We report in this paper the purification and some properties of yeast ferrochelatase. Although ferrochelatase from various origins has already been purified (1-5), no information has been available concerning the biogenesis of this mitochondrial membrane-bound enzyme. Preparation of the purified enzyme and antibodies raised against it were used to show that yeast ferrochelatase is synthesized as a higher molecular weight polypeptide (Mr = 44,000) (i) from in vitro translation of total yeast RNAs and (ii) in vivo, where it is rapidly processed to the mature form of the enzyme (Mr = 40,000). The need for two immunoprecipitations to isolate pure ferrochelatase labeled in vivo, together with the great homogeneity of the electrophoretic profiles of the associated proteins, suggests that ferrochelatase is part of a multienzymatic complex which is not completely dissociated by SDS treatment. This seems all the more probable since Taketani et al. (32) have recently shown that ferrochelatase is associated with complex I of the bovine mitochondrial electron transport chain. Such complexes might be physiologically related to the ferrous iron requirement of ferrochelatase. These complexes could be in-
volved either in the reduction of ferric iron to ferrous iron or in keeping iron under reducing conditions. The anti-ferrochelatase antibodies seem to be monospecific, based on the results obtained by Western blotting and immunoprecipitation of in vitro translated ferrochelatase.

The mature mass of ferrochelatase was found to be identical to that of wild-type strains in all the heme-deficient yeast strains assayed (except those deficient in ferrochelatase protein). This was true for both the in vitro translation products and the mature enzyme detected by immunodecoration after SDS-PAGE of trichloroacetic acid-precipitated total protein extracts. These results do not favor the involvement of heme in the processing of the precursor form of ferrochelatase as described for one of the steps of proteolytic maturation of imported cytochrome c1 (19).

Contrary to what was described for the mouse liver (34) or the human liver (5) ferrochelatase, the N-terminus of purified yeast ferrochelatase was accessible to Edman degradation. Thus, the N-terminal amino acid sequence of the mature yeast ferrochelatase has been determined to over 20 residues. A GeneBank™/EMBL libraries search has revealed no sequence homology to previously cloned genes or peptide sequences. Our data will be necessary for localizing the cleavage site of the precursor once the complete structure of the protein (through gene sequence analysis) is available. It is interesting to note that the N-terminal sequence of the protein is frayed. Similar results have been reported for the α, β, and γ subunits of the F1-ATPase from bovine liver mitochondria (35) and for the inhibitor of the F1-ATPase of the same origin (36). All these proteins are encoded by nuclear genes and synthesized as higher molecular mass polypeptides. The maturation of mitochondrial precursor proteins is catalyzed by a metallocprotease located in the matrix space of mitochondria (37); no consensus cleavage sequence has been reported so far. Our results suggested that this protease may recognize both the N- and C-terminal sides of the amino acids involved in the cleavage site of ferrochelatase. However, artifactual proteolytic degradation of ferrochelatase during the process of isolation of the enzyme cannot be completely excluded.

The heterogeneity of the N-terminal sequence of yeast ferrochelatase favors the existence of an N-terminal extension of the precursor which is cleaved off during the process of importation into the mitochondria. However, we cannot exclude the possibility of a concomitant C-terminal extension, as recently demonstrated for the COX 9 and COX 8 gene products (38, 39).

Yeast ferrochelatase is, in many aspects, similar to the enzyme from higher eukaryotes. The relative molecular weight of the mature yeast ferrochelatase is close to that reported for the vertebrate enzymes (Mw = 40,000-42,000), but is very different from that of the bacterial enzyme (Mw = 105,000; R. spheroides; Ref. 8). The kinetic properties of the yeast enzyme were also very similar to those of the rat or bovine enzyme. The Km values we obtained for protoporphyrin IX, ferrous iron, and zinc are somewhat lower than those previously described, possibly because of differences in assay accuracy and sensitivity. It is important to note that all our kinetic studies have been performed using the physiological substrate of the enzyme, protoporphyrin IX, and not the more hydrophilic nonphysiological dicarboxylic porphyrins, mesoporphyrin IX or deuteroporphyrin IX. Thus, the kinetic constants determined under our conditions are probably close to those prevailing in vivo. A random-order equilibrium mechanism of the ferrochelatase reaction was consistently found, with the value of K<sub>n</sub> (protoporphyrin) = 9 x 10<sup>-8</sup> M being identical when measured in both the iron-chelatase and zinc-chelatase assay systems. The occurrence of contaminating metals (mainly zinc) in all chemicals generated many pitfalls in the measurement of the kinetic parameters of zinc-chelatase and iron-chelatase activities of ferrochelatase, as already described for the yeast, human, and chicken membrane-bound ferrochelatases (13, 14). We previously postulated that the synthesis of zinc-protoporphyrin in vivo when yeast resting cells are incubated in the presence of oxygen and in the absence of glucose might be related to a decrease in ferrous iron concentration, zinc and ferrous iron being competing substrates for ferrochelatase (6). The differential affinity we describe in this paper of ferrochelatase for zinc in the presence or absence of DTT could mimic a situation of physiological relevance involved in ferrochelatase activity control. This hypothesis is consistent with the fact that, as already described for the rat liver and the chicken erythrocyte enzymes (1, 7) and studied in detail for the enzyme isolated from bovine liver mitochondria (3), sulfhydryl groups are involved in ferrochelatase activity.

As has been shown for the rat liver enzyme (1), but not for the bovine enzyme (2), fatty acids were necessary for maximum activity of the purified yeast enzyme. The fatty acids tested (myristic acid, palmitic acid, and oleic acid) were almost equally efficient in activating yeast ferrochelatase. However, some nonenzymatic zinc-protoporphyrin formation was observed with oleic acid (10% of enzymatic rate), whereas the oleic acid-containing detergent Tween 80 did not catalyze such synthesis. An attempt was made to develop an affinity chromatography support by linking palmitic acid to Affi-Gel 102 through 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl cross-linking to obtain more information on the role of fatty acids in ferrochelatase activity. The enzyme did not bind to the gel under the buffer conditions needed for maximum in vitro ferrochelatase activity. It is highly possible, as suggested by Taketani and Tokunaga (1) and others (19, 33, 40, 41), that the free carboxylic moiety of fatty acids is needed for activating ferrochelatase. This problem will be studied by using fluorescent derivatives of palmitic acid.

One of the heme-deficient yeast strains isolated in our laboratory (G214), characterized by an accumulation and excretion of protoporphyrin IX and a lack of ferrochelatase activity in vivo, has been reported to carry a single nuclear recessive mutation (15). However, genetic analysis of the segregation of the mutated character revealed that the lack of ferrochelatase activity in this strain may possibly result from two nuclear mutations: one, heml5-1, affecting the structural gene of ferrochelatase and another affecting the expression of the mutation. A high frequency of partial reversion in strain G214 was also observed with accumulation of protoporphyrin IX despite synthesis of heme in the revertants (wild-type strains do not accumulate protoporphyrin) (17). Another ferrochelatase-deficient strain of yeast isolated in our laboratory (G231) is phenotypically very stable. The antibodies against yeast ferrochelatase were used to characterize these ferrochelatase-deficient strains (G214 and G331) better. Both are devoid of immunoreactive protein detectable either in vivo or from in vitro translatable mRNA. A nucleotide probe will be required 1) to determine the actual amount of ferrochelatase mRNA and/or its half-life in wild-type and mutant strains and 2) to characterize the mutated alleles in these two strains.

The tools are now available to study the topology of yeast ferrochelatase within the inner membrane of mitochondria and to determine the possible interactions with other polypeptide chains, especially protoporphyrinogen oxidase. This will provide us with a better understanding of the functioning of this metabolically important coupled system.
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